The Metabolic Pathway of Propionate in *Euglena gracilis* z Grown under Illumination

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*Euglena gracilis* grew with propionate as the sole carbon source under illumination but not in the dark. The metabolic pathway of propionate was determined in *E. gracilis* growing under illumination. Labeled propionate was first recovered in the amino acid fraction and gradually transferred to protein and paramylon with a lapse in incubation time. In the amino acid fraction aspartic acid was most densely labeled initially, followed by glutamic acid indicating that propionate is metabolized via succinate. The result agreed with the radiorespirometric pattern of specifically labeled propionates: 1-C ꕳ 2-C ꕳ 3-C. *E. gracilis* contained propionyl-CoA carboxylase, but not α-hydroxyglutarate synthase. The reaction product of the enzyme was identified as methylmalonyl-CoA. The enzyme activity increased in the log phase when propionate in growth medium was actively utilized for growth. From these results we have concluded that illuminated *Euglena* metabolizes propionate through propionyl-CoA and methylmalonyl-CoA to tricarboxylic acid cycle intermediates for growth.

\[\text{Materials and Methods}\]

*Organism and culture.} *E. gracilis*, strain z, was cultured in the Cramer-Myer's medium\(^2\) supplemented by sodium propionate (6.6 g/liter) under illumination (3000 lux) at 27°C. By culturing cells in this propionate medium several times in succession, they were adapted and achieved a satisfactory growth.

\[\text{Determination of propionate in the culture medium.}\] *Euglena* cells were removed from the culture medium by centrifugation at 5000 x g for 5 min, and the cell-free medium was used for the determination of the propionate, which remained unused, by gas-liquid chromatography (Yanagimoto, Type GC 550 FP) on a stainless steel column (0.3 x 75 cm) packed with 15% diethylene glycol succinate (DEGS) on 60 ~ 80 mesh NEOPAK AS (Nishino Ind. Tokyo). The column, injection and detection temperatures were 172, 230 and 260°C, respectively. The flow rate of carrier nitrogen gas was 30 ml/min.

\[\text{Feeding experiment of labeled propionates.}\] The cells grown for 6 days in the propionate-mineral medium under illumination were washed with 10 mM sodium phosphate buffer (pH 6.8) and suspended in the same buffer (1.12 x 10\(^7\) cells/ml). The cells in 2 ml of the suspension were incubated with 0.5 µCi of 1\^-C or 2\^-C-propionate (48 mCi/mmol and 47.7 mCi/mmol, respectively) at 27°C for 5 to 60 min under illumination (3000 lux). The radioactivity in the evolved CO\(_2\) was determined by the technique of Yokota et al.\(^3\). For the determination of the radioactivity distributed among cell components, feeding was stopped by prompt cooling of the feeding mixture in an ice bath prior to collecting cells, and cell fractions were obtained as follows. The fed cells were washed with chilled saline solution and extracted 4 times with 2 ml each of hot 80% ethanol. The extracts were combined and defatted 3 times.
with 8 ml each of cyclohexane. The cyclohexane-soluble fraction was used as the lipid fraction. The cyclohexane-insoluble fraction was evaporated to dryness, and the residue was dissolved in 2 ml of water and passed through a column of Dowex 50 (H⁺ form). Amino acids were eluted with 1 N aqueous ammonia from this column. The effluent from the column was applied onto a Dowex 1 × 8 (HCOO⁻ form) column, and the effluent from this column was used as the sugar fraction, and the eluate with 1 N formic acid as the acid fraction. Protein and paramylon were obtained from the residue of the ethanolic extraction and were determined according to Yokota et al.3)

Distribution of radioactivity among individual free amino acids was determined after similar incubations for 0.5 to 15 min. Feeding was stopped by adding perchloric acid, and the feeding mixture was sonicated (10 Kc) for a total of 2 min with 3 intervals of 30 sec each and centrifuged at 10000 × g for 10 min. The supernatant was loaded onto a Dowex 50 (H⁺ form) column, and the eluate with 1 N aqueous ammonia was subjected to chromatography on an Aminex A-5 column (BIO-RAD Labs.)

Radiorespirometric measurements of CO₂ evolved from propionates labeled at different positions. A 2-ml suspension of cells (2.31 × 10⁷ cells) was incubated with 0.1 μCi of 1-¹⁴C-, 2-¹⁴C- or 3-¹⁴C-propionate with the same specific radioactivity (4.77 mCi/mmol). Determination of radioactivity in the evolved CO₂ was carried out as described above.

Enzyme assays. A crude enzyme solution was prepared from E. gracilis grown on propionate under illumination by the method of Yokota et al.3) Propionyl-CoA carboxylase (EC 6.4.1.3) was assayed by measuring the propionyl-CoA-dependent ¹⁴C-bicarbonate fixation by the method of Knight.4) α-Hydroxyglutarate synthase (EC 4.1.3.9) was assayed by the method of Wegener et al.5) and NAD-linked and NADP-linked succinic semialdehyde dehydrogenase (EC 1.2.1.16), by that of Tokunaga et al.6) Isocitrate lyase (EC 4.1.3.1) was assayed by the method of Dixon and Kornberg.7)

Protein determination. The method of Lowry et al.8) was followed.

Chemicals. Propionyl-CoA was prepared by the method of Simon and Shemin.9)

RESULTS

Growth of E. gracilis on propionate under illumination

E. gracilis which had been adapted to propionate grew on propionate as the sole organic carbon source under illumination to give a cell population of 10 × 10⁶/ml in the stationary phase, which was reached after 6 to 8 days of culturing, like on other added carbon sources. The attained cell population was 10 times as great as the population in photoautotrophic cultivation without the addition of propionate, but was about 50% of the population in the Koren–Hutner medium3) in which glucose and glutamic acid were major carbon sources. Unadapted cells numbered 2 × 10⁶/ml after 10 days of culturing in the propionate-supplemented mineral medium. Propionate in the medium was eliminated almost completely during the culturing of adapted E. gracilis.

Propionate was also eliminated by E. gracilis but did not support the protozoan growth in the dark.

Incorporation of radioactive propionate by E. gracilis and distribution of radioactivity among cell components

E. gracilis adapted to propionate was incubated for 5 to 60 min with 1-¹⁴C- or 2-¹⁴C-propionate under illumination. Table I shows that the labeled propionates are taken up by the cells progressively with the advance of incubation, and that with 1-¹⁴C-propionate greater parts of the taken up label are released as labeled CO₂ while with 2-¹⁴C-propionate distribution of label to released CO₂ is much less. Release of labeled CO₂ is less along with incubation with either compound.

Of the label retained in cell components, the highest percentage is found in the amino acid fraction, particularly after a short incubation with either propionate; the radioactivity retained in the amino acid fraction, particularly after a short incubation with either propionate; the radioactivity retained in the amino acid fraction after incubation for 5 min was 42% with 1-¹⁴C-propionate and 44% with 2-¹⁴C-compound of the whole radioactivity retained in the cell components. The label in the protein and paramylon fractions increased, particularly markedly in the latter, with the time of incubation with both compounds. The results indicate that the propionate taken up and retained in cell materials is largely converted first into amino acids probably through the precursor organic acids.
TABLE I. DISTRIBUTION OF RADIOACTIVITY AMONG CELL FRACTIONS AFTER INCUBATION OF E. gracilis WITH 1-14C- AND 2-14C-PROPIONATES

The figures are percentages of the total radioactivity taken up in each experiment.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1-14C-Propionate</th>
<th></th>
<th>2-14C-Propionate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>15 min</td>
<td>60 min</td>
<td>5 min</td>
</tr>
<tr>
<td>CO₂</td>
<td>72.3</td>
<td>64.7</td>
<td>53.2</td>
<td>30.4</td>
</tr>
<tr>
<td>Protein</td>
<td>4.6</td>
<td>8.3</td>
<td>9.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Paramylon</td>
<td>0.6</td>
<td>7.8</td>
<td>15.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.8</td>
<td>1.0</td>
<td>2.3</td>
<td>12.6</td>
</tr>
<tr>
<td>Amino acid</td>
<td>11.5</td>
<td>7.7</td>
<td>8.7</td>
<td>30.8</td>
</tr>
<tr>
<td>Organic acid</td>
<td>3.1</td>
<td>5.0</td>
<td>4.6</td>
<td>6.0</td>
</tr>
<tr>
<td>Sugar</td>
<td>7.1</td>
<td>5.5</td>
<td>5.6</td>
<td>12.0</td>
</tr>
<tr>
<td>Total radioactivity taken up (cpm × 10^-3)</td>
<td>8.4</td>
<td>54.0</td>
<td>119.0</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Distribution of radioactivity among amino acids after incubation with labeled propionate

The radioactivities in aspartic acid, glutamic acid and alanine, the diagnostic amino acids for the metabolic pathways, after 0.5 to 15 min of incubation of E. gracilis with 2-14C-propionate under illumination are shown in Table II. The amounts of these amino acids in the amino acid pool were virtually constant throughout the incubation being about 0.21, 0.62 and 0.45 nmol/10⁶ cells, respectively. The distribution of radioactivity to aspartic acid was the largest initially but decreased with the lapse of time, while that to glutamic acid increased gradually along with the incubation to reverse the order after 2 min. The ratio of specific radioactivity between aspartic acid and glutamic acid was 1:0.21 after 0.5 min, which varied to 1:0.66 after 2 min, 1:0.9 after 5 min and 1:1.17 after 15 min of incubation. The distribution of the label to alanine was relatively little, and its specific activity ratio to aspartic acid was hardly changed throughout the incubation. Incorporation of the label of propionate into other amino acids was not significant.

Radiorespirometric patterns of 14CO₂ from 1-14C-, 2-14C- and 3-14C-propionates

Figure 1 shows the time course of evolution of labeled CO₂ when E. gracilis was incubated with 1-14C-, 2-14C- or 3-14C-propionate under illumination up to 30 min. The radiorespirometric pattern was 1-14C > 2-14C = 3-14C.

TABLE II. CHANGES OF SPECIFIC RADIOACTIVITIES OF ASPARTIC ACID, GLUTAMIC ACID AND ALANINE IN THE AMINO ACID POOL IN E. gracilis INCUBATED FOR 0.5 TO 15 MIN WITH 2-14C-PROPIONATE

Figures in parentheses are percentages of the total label in the amino acid pool in each experiment.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Specific radioactivity (cpm/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 min</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>255 (25.2)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>54 (15.4)</td>
</tr>
<tr>
<td>Alanine</td>
<td>49 (10.1)</td>
</tr>
</tbody>
</table>
Enzyme studies

Activities of some enzymes possibly participating in the metabolic pathways of propionate giving succinate as an intermediate in *E. gracilis* grown on propionate under illumination were determined. α-Hydroxyglutarate synthase was not detected in Euglena cells. In the propionate-grown cells, isocitrate lyase showed an activity of 0.067 nmol/10^6 cells/min while in the glucose-grown cells the activity was 0.135 nmol/10^6 cells/min.

The activity of NAD-linked succinic semialdehyde dehydrogenase in the extract of *E. gracilis* grown on propionate was 35.3% and 60.3%, respectively, of those of the cells grown on glucose and grown photoautotrophically, and that of NADP-linked succinic semialdehyde dehydrogenase was 52.6% and 77.6%, respectively.

The extract of *E. gracilis* grown on propionate contained a considerable activity of propionyl-CoA carboxylase, which had an optimum pH at 8.5 and was dependent on Mg^{2+}, ATP, propionyl-CoA and glutathione like the same enzyme in other organisms.\(^4,10,11\)

Methyllumonate, derived from methylmalonyl-CoA, the reaction product of the enzyme, was identified in the reaction mixture. A mixture of propionyl-CoA, \(^{14}\)C-bicarbonate and an *Euglena* extract was incubated according to Knight,\(^4\) and, after hydrolysis with potassium hydroxide, the mixture was charged onto a column of Dowex 1×8 (HCOO⁻ form). Methyllumonate was eluted with 1N formic acid and identified by paper chromatography with the solvent systems of n-butanol-acetic acid-water (12:3:5) and n-butanol-formic acid-water (4:1:2). Approximately 80% of the radioactivity in the whole formic acid eluate was recovered as radioactive methylmalonate (Fig. 2), which was not formed in the absence of propionyl-CoA in the reaction mixture.

Methyllumonate was also identified by gas-liquid chromatography. An eluate from the Dowex 1×8 column was evaporated to dryness, and the residue was dissolved in a small volume of acetone and submitted to a gas-liquid chromatographic apparatus (Gas Chromatography Ind., model KOR-70) at
FIG. 3. Change of the Activity of Propionyl-CoA Carboxylase of *E. gracilis* along with Growth under Illumination.

90°C with a glass column (100 x 0.2 cm) packed with 15% DEGS. The product of the enzyme reaction showed a retention time which was identical with that of the authentic methylmalonate.

In the *E. gracilis* cells actively taking up propionate, the activity of propionyl-CoA carboxylase was, as assayed in the cell extract, 0.418 nmole of bicarbonate fixed per min per 10⁶ cells. This enzyme activity in the *Euglena* cells grown on propionate was 3 times as high as that in the cells grown photoautotrophically and 5 times as high as that in those grown on glucose. The enzyme showed a peak of activity in the late logarithmic phase of growth, followed by a gradual decrease during the stationary phase (Fig. 3).

**DISCUSSION**

*E. gracilis*, strain z, was demonstrated to grow on propionate as the sole carbon source. Adaptation was required for the cells to grow extensively on the acid; the attained cell population was 50% of that in the rich, glucose-glutamate medium. Unadapted cells gave a poorer growth after longer culturing. A similar but more remarkable adaptation was found in *E. coli* for the growth on propionate; unadapted cells required a long lag.¹²

The growth of *Euglena* cells on propionate occurred only under illumination, and although the acid was also taken up by the cells it did not support the protozoan growth in the dark. Similar "photoassimilation" has been reported for acetate, glycolate, glycine and serine in *Euglena*,¹³,¹⁴ and also for some organic compounds in several species of photosynthetic microorganisms.¹⁵⁻¹⁷ The fate of propionate in *E. gracilis* in the dark and the regulative mechanism of the photoassimilation are the subjects of our forthcoming papers.

Propionate is known to be metabolized in mammalian tissues, higher plants and many microorganisms, and several pathways have been reported for its metabolism¹⁸ (Fig. 4). The most studied pathway, operative in mammalian tissues and in some bacteria, involves fixation of bicarbonate to yield succinate via methylmalonyl-CoA. Another pathway producing succinate involves condensation of propionyl-CoA and glyoxylate to yield α-hydroxyglutarate as an intermediate. In higher plants and many other species of bacteria, propionyl-CoA is first dehydrogenated into acryl-CoA, which is converted into lactyl-CoA and then acetyl-CoA via pyruvate, or into (β-hydroxypropionyl-CoA which is converted into acetyl-CoA via malonic semialdehyde or malonyl-CoA. Recently a new pathway was found in *Candida lipolytica*,¹⁹,²⁰ in which

![Diagram of metabolic pathways](image-url)
propionyl-CoA condenses with oxaloacetate to give methylcitrate which is cleaved into pyruvate and succinate.

To determine which pathway *E. gracilis* follows to metabolize propionate, distribution of the label among cell components after incubation with labeled propionates was first studied (Table I). Considerable parts of the label were released as labeled CO2 rapidly after the cells took up labeled propionates. The release was more extensive with 1-14C-propionate than with 2-14C-propionate, indicating the carboxyl carbon is more readily decarboxylated than are other carbons. Among the cell components, early distribution of the label was found most abundantly in amino acids, suggesting that the propionate taken up and retained in *Euglena* cells is first converted into amino acids via precursor organic acids.

As shown in Table II, aspartic acid was most densely labeled immediately after the start of incubation of *E. gracilis* with labeled propionate. The label of aspartic acid decreased along with the incubation while that of glutamic acid increased to surpass the label of aspartic acid soon. Initial labeling of aspartic acid suggests that propionate is converted into succinyl-CoA or succinate for entering into the TCA cycle in *Euglena* cells. If propionate enters into the TCA cycle after being metabolized to acetyl-CoA, glutamic acid must be labeled earlier than aspartic acid, but this was not the case. A very low isocitrate lyase activity in the propionate-grown cells supports the conclusion.21) Labeling of alanine was relatively low and unchanged during the incubation suggesting that pyruvate is not the key intermediate.

Wegener *et al.*22) found that the radiorespirometric pattern of 14C from 1-14C-, 2-14C- and 3-14C-propionates was helpful for determining the metabolic pathway of propionate. The expected patterns with the key intermediates are as follows: 1 > 2 > 3 lactate, 1 > 3 > 2 α-hydroxypropionate, 3 > 1 > 2 malonic semialdehyde, 1 > 2 = 3 methylmalonyl-CoA and α-hydroxyglutarate. Experimental results (Fig. 1) showed that the pattern in *E. gracilis* was 1 > 2 = 3, indicating that the pathway is through succinate via either methylmalonyl-CoA or α-hydroxyglutarate.

The methylcitrate pathway19,20) may be excluded since it yields pyruvate as well as succinate, and the labeling pattern of alanine does not support the production of pyruvate as an intermediate.

To decide which of the methylmalonyl-CoA and α-hydroxyglutarate pathways *E. gracilis* employs, key enzymes involved in these metabolic routes were determined. The α-hydroxyglutarate synthase activity was not detected at all in the extract of *E. gracilis* grown on propionate under illumination. The activity of isocitrate lyase which should supply glyoxylate for the conversion of propionyl-CoA into α-hydroxyglutarate was much lower in the *E. gracilis* cells grown on propionate than in the cells grown on glucose, a repressor of glyoxylate cycle23,24) indicating that the glyoxylate cycle is not significantly operative. The activities of both NAD-linked and NADP-linked succinic semialdehyde dehydrogenases, which may also participate in the α-hydroxyglutarate route, in the propionate-grown *E. gracilis* cells were considerably lower than those in *Euglena* cells grown on glucose and grown photoautotrophically. These results indicate that propionate is not metabolized via α-hydroxyglutarate in *E. gracilis* grown on propionate.

*Euglena* cells contained a considerable activity of propionyl-CoA carboxylase which converts propionyl-CoA into methylmalonyl-CoA, and methylmalonyl-CoA was not formed in the absence of propionyl-CoA. This enzyme was induced by growing *E. gracilis* on propionate. Identification of methylmalonate, derived from methylmalonyl-CoA, the key intermediate of the methylmalonyl-CoA pathway, by paper and gas-liquid chromatographies has established that in illuminated *E. gracilis* propionate is metabolized through propionyl-CoA, methylmalonyl-CoA, succinyl-CoA and succinate, which enters into the TCA cycle. That the activity of this enzyme was the highest when the consumption of propionate by *Euglena* cells was most vigorous.
supports that propionate is truly metabolized in *E. gracilis* by the methylmalonyl-CoA pathway.

Metabolism of propionate by the methylmalonyl-CoA pathway well explains the results of feeding experiments. Since propionate is converted into succinate to enter the TCA cycle, the carboxyl carbon of propionate is decarboxylated sooner than the methyl and methylene carbons which are therefore retained more in lipids, free sugars, paramylon and proteins.

REFERENCES